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AMINO ACID COMPOSITION OF β-CASEINS FROM THE MILKS OF *BOS INDICUS* AND *BOS TAURUS* COWS: A COMPARATIVE STUDY

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(*Received* 10 *December* 1968)

Abstract—1. β-Caseins B (*Bos taurus*), B_Z and D (*Bos indicus*) were examined for amino acid composition.

2. B_Z differs from B in content of several neutral amino acids and possibly glutamic acid, as well as in tryptic "fingerprints".

3. β-D differs from B_Z notably in content of arginine (+1), lysine (+1) and histidine (−1), but also in one additional residue each of threonine, serine and alanine.

4. β-D (like β-C, *Bos taurus*) lacks 1–2 residues of phosphorus as compared with B.

5. It is likely that β-C (*Bos taurus*), itself, occurs as polymorphs.

INTRODUCTION

IN AN EARLIER communication, Aschaffenburg *et al.* (1968) reported that the caseins of the milks of Indian and African Zebu (*Bos indicus*) cows, like those of Western (*Bos taurus*) breeds of cattle, showed genetic differences in α_{s1}-, β- and κ-caseins. Of particular interest were the observations that a new variant of the β-casein series, D, was present in Zebu but absent in Western cattle, and that β-casein B from Zebu (termed B_Z) was not identical to Western β-B in either amino acid composition or tryptic peptide fingerprinting. However, β-casein A^2 and α_{s1}-caseins B and C obtained from Zebu milks were, in fact, identical in peptide mapping to their counterparts in Western milks. These observations, made in exploratory experiments, prompted us to investigate thoroughly the differences in amino acid composition and to compare the tryptic peptide fingerprints of Western β-caseins B and C, and B_Z and D (Zebu).

MATERIALS AND METHODS

Casein samples

β-Casein B was obtained from a Jersey cow homozygous for the B-gene and β-C (a gift from Dr. R. Aschaffenburg) was from a Guernsey cow homozygous for the C-gene. β-Caseins B_Z and D were obtained as the heterozygote B_ZD mixture from Dr. A. Sen, Calcutta, India.

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Purification of β-casein variants

β-Caseins B and C were first concentrated by urea-fractionation of whole casein by the method of Aschaffenburg (1963). κ-Casein and α_{s1}-impurities were removed by column chromatography of about 500 mg crude protein on DEAE–cellulose, 3·3 M urea, buffered at pH 7·0 with imidazole–HCl (0·01 M), in the presence of 2-mercaptoethanol by the method of Thompson (1966). A linear gradient of 0–0·25 M NaCl was used to elute the casein components. With a flow rate of 100 ml/hr, elution of β-casein was complete in about 10 hr. The β-casein was dialyzed against deionized water at 4°C until free of urea and salt and then lyophilized.

Because homozygous β-D was unavailable, B_Z and D were separated from a B_Z–D mixture by DEAE–cellulose chromatography (as described above) of 1 g of crude casein. Resolution of B_Z from D was complete and the two were than rechromatographed to remove trace impurities.

Gel electrophoresis

The purity of β-casein preparations was verified by polyacrylamide-gel electrophoresis of the proteins (5 mg/ml) in 7% Cyanogum, 4·5 M urea, Tris-citrate buffer at pH 9·1 in the presence of 2-mercaptoethanol (Thompson, 1966).

Tryptic digestions and peptide mapping

Twenty mg of each β-casein variant was digested at 37°C with trypsin (enzyme–substrate ratio, 1 : 100) in a pH-stat at pH 8·0. The pH was maintained by the automatic addition of 0·1 N NaOH. Shortly after the addition of trypsin the solution became turbid, a condition which persisted for about 10 min before clearing. Following reaction for 4 hr, the digests were shell frozen and lyophilized.

Peptide mapping, as for α_{s1}-casein (Kalan *et al.*, 1966), was achieved by a two-dimensional combination of high-voltage electrophoresis and chromatography, with 3-mg samples of each β-casein digest. Electrophoresis was carried out on Whatman No. 3 MM paper for 2 hr at 40 V/cm in a horizontal water-cooled plate apparatus; pyridine–acetic acid–water buffer at pH 6·4 was used (Ingram, 1958). After air-drying, the papers were subjected to ascending chromatography in the second dimension with the solvent system, *n*-butanol–acetic acid–water (3 : 1 : 1). The chromatograms were developed with 0·2% ninhydrin in acetone at 110°C.

Amino acid analysis

Analyses for amino acids were made with automatic recording equipment using procedures described by Moore & Stein (1963). Weighed samples (about 2 mg) of the proteins were hydrolyzed in 1-ml portions of glass-distilled 6 N HCl in sealed, evacuated tubes maintained at 110°C for periods of 24, 72 and 96 hr. At least two samples of each protein were hydrolyzed at each period. Thereafter, the hydrolysates were handled as described by Moore & Stein.

Tryptophan content

Determination of tryptophan was made by the method of Spies (1967) and we are grateful to Dr. Spies for these analyses.

Phosphorus content

Phosphorus analyses were run in duplicate on 10-mg samples of β-caseins (equilibrated to constant-moisture content) by the wet digestion method of Sumner (1944).

RESULTS

Purification of β-caseins

Figure 1 demonstrates the separation of β-casein B_Z from β-casein D by column chromatography on DEAE–cellulose, 3·3 M urea. Clearly, the two

phenotypes are resolved from each other and, as Fig. 2 illustrates, are essentially free of α_{s1}-caseins and κ-caseins. The trace impurities (migrating faster than the B_Z and D variants) were removed by an additional rechromatography of the samples. Unfortunately to date we have been unable to procure a homozygous

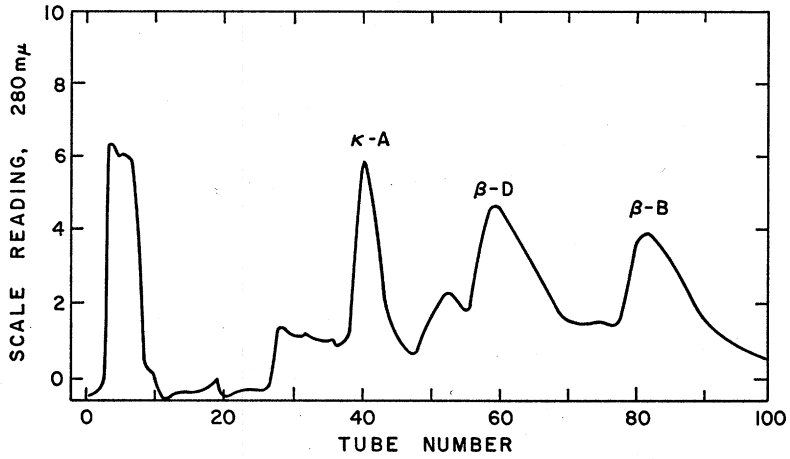

FIG. 1. DEAE–cellulose chromatography of a mixture of β-caseins B_Z and D in 3·3 M urea, 0·01 M imidazole–HCl buffer plus 2-mercaptoethanol. One g protein charge. α_{s1}-Casein region omitted.

β-casein D but separation of the heterozygotes is relatively simple. Of interest is the fact that in separation of the β-B_ZD heterozygote, the concentration of β-D is found to be somewhat greater than that of β-B_Z, that is, in a 60 : 40 ratio. In our studies on the rare α_{s1}-casein A mutant, it was found in a 40 : 60 ratio in the AB or AC heterozygotes (Thompson *et al.*, 1969).

Phosphorus contents

The phosphorus contents of β-A^2, B, B_Z and D were 0·57, 0·55, 0·55 and $0{\cdot}40 \pm 0{\cdot}01$ per cent, respectively. These values correspond to 5 g atoms of phosphorus per mole for A^2, B and B_Z and 3–4 g atoms for D using a molecular weight value of 24,000 Daltons.

Amino acid composition

Many of the data which have been reported for the amino acid composition of polymorphic milk proteins (almost all being the results of analyses of proteins prepared from milks of Western breeds of cattle) have been summarized by McKenzie (1967). Differences in composition among the genetic variants of each of the components of casein, α_{s1}, β and κ, and of β-lactoglobulin have been found to be, most often, single or double amino acid substitutions. α_{s1}-Casein is an exception to this generalization; the A variant lacks a number of amino acids found to be present in α_{s1}-caseins B and C (Gordon *et al.*, 1965). It is also true that,

while there is good agreement in the results of amino acid analyses from different laboratories for most of the polymorphic milk proteins, there is less satisfactory agreement in the case of the variants of β-casein. Thus, the exact amino acid differences distinguishing β-caseins A^1, A^2, A^3, B and C are not yet clearly established. This is due, in part, to the fact that only recently has the polymorphism of β-casein A been detected (Kiddy *et al.*, 1966; Peterson & Kopfler, 1966); in part, to the low gene frequency of β-casein C (Thompson *et al.*, 1964) and the problem of obtaining pure preparations of this polymorph; and finally to the experimental difficulties inherent in the accurate determination of high concentrations of certain amino acids, notably glutamic acid and proline, characteristic of β-casein.

The results of our analyses of β-casein B (Jersey) and β-caseins B_Z and D (Zebu) are shown in Table 1. The amino acid composition of β-B agrees very well with the previously published analyses of Pion *et al.* (1965) and Peterson *et al.* (1966). Of interest is that each group (ours included) has had trouble in pinpointing the exact number of glutamic acid (38–40) and proline (32–35) residues per molecule of protein, but this is not surprising in view of the large number of each.

In comparing β-B with β-B_Z it is evident that they differ somewhat in amino acid composition despite the fact that their electrophoretic mobilities in both acid and alkaline media are indistinguishable. To be sure, the differences in content of threonine, serine, proline, alanine, valine, methionine, leucine and phenylalanine do not affect the net charge of the molecule and there is no difference in content of phosphoric acid residues. On the other hand, there is probably a significant difference in content of glutamic acid. This is not reflected in a difference in mobility. Unfortunately, reliable analyses for amide groups have not been made for these proteins and without this information there is no way to account for the apparently identical net charge. Assuming that β-caseins B and B_Z contain 33 proline residues per 24,000 molecular weight, the total number of amino acid residues in each is 205 or 206 and 201–203, respectively. On the same basis β-casein D contains 204–206 per molecule, but comparison of residue numbers in β-caseins B and D, while indicating a general similarity in relative percentages of amino acids, shows that most are present in different amounts.

Examination of the amino acid compositions of β-B_Z and β-D caseins reveals, again, numerous differences. It may be argued, perhaps, that this pair is more nearly alike than β-B and β-B_Z in that significant differences are evident for fewer amino acids, namely, lysine, histidine, arginine, threonine, serine, proline and alanine, and that these vary by only one residue. Of greater interest are the several differences in charged groups. Again, estimates of amide groups are not available, but here clear differences in basic amino acid content occur, and, of overriding importance to the net charges of the molecules, the unequal amounts of phosphorylated amino acids should be emphasized. The lower electrophoretic mobility at alkaline pH values of β-casein D is undoubtedly a reflection of its lower content of phosphorus.

Also included in Table 1 for purposes of comparison is our best estimate, based on numerous analyses of two separate preparations, of the amino acid

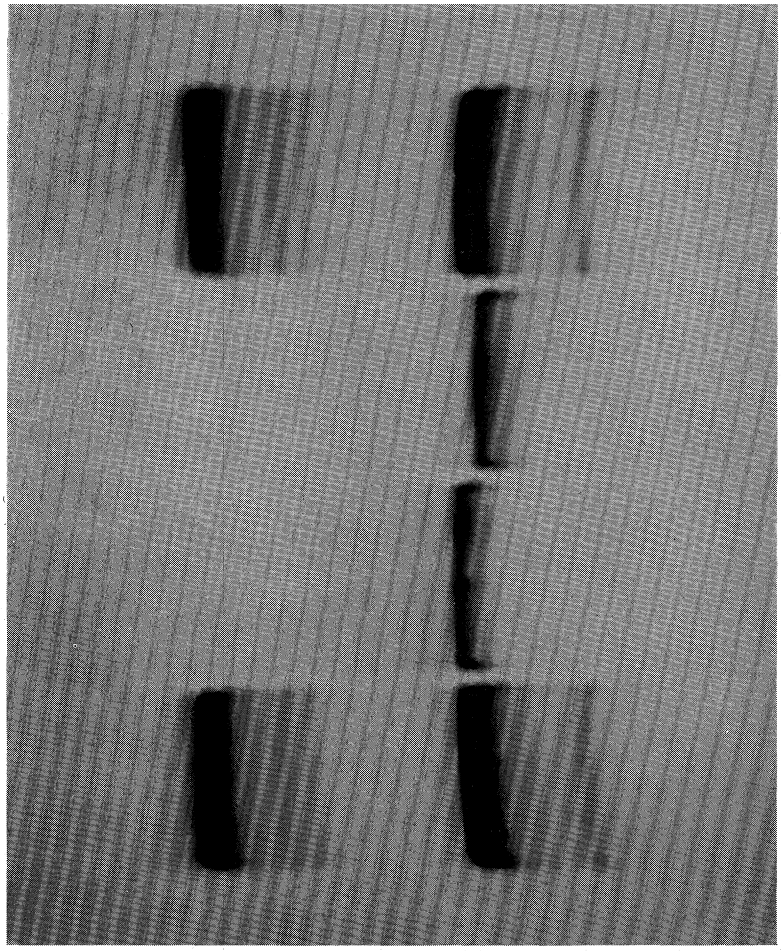

FIG. 2. Purity of β-caseins B_Z and D after a single pass over DEAE–cellulose (Fig. 1) as shown by polyacrylamide-gel electrophoresis, pH 9·1, 4·5 M urea. Upper and lower patterns represent the whole casein from which β-D (next to top) and β-B_Z (next to bottom) were isolated.

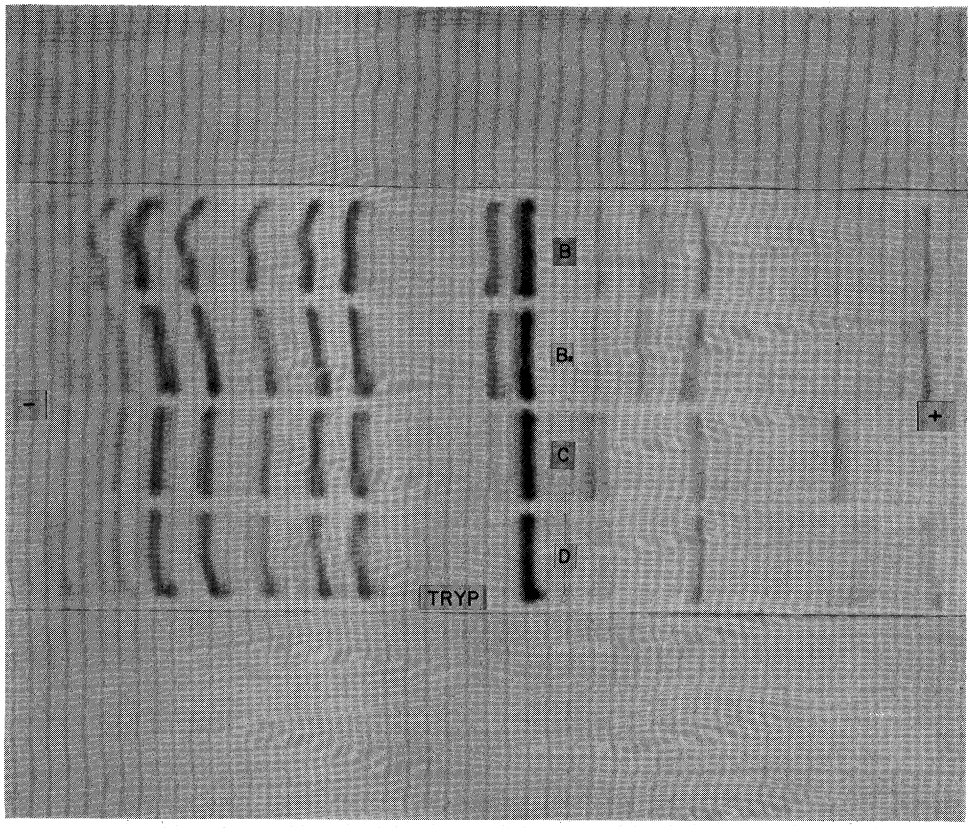

FIG. 3. High-voltage electrophoresis of tryptic digests of β-caseins B, B_Z, C and D. Four mg hydrolysate applied. See text for details.

composition and phosphorus content of the sample of β-casein C used in this investigation. Detailed analytical data will be reported elsewhere. They show unequivocally that this form of β-casein C differs appreciably in content of a number of amino acids from the samples analyzed by Pion *et al.* (1965) and by Peterson *et al.* (1966). Disregarding small differences of questionable significance in glutamic acid, proline, valine and leucine content, our β-casein C differs from

TABLE 1—AMINO ACID COMPOSITION OF β-CASEINS (RESIDUES OF AMINO ACID PER MOLECULE PROTEIN)

	Found*			Estimated whole number residues			
	B	B_Z	D	B	B_Z	D	C
Lys	10·9 (10)†	10·9 (9)	11·7 (9)	11	11	12	12
His	6·0 (10)	5·7 (9E)‡	5·0 (9E)	6	6	5	6
Arg	4·9 (10)	4·6 (9)	3·8 (9)	5	5	4	4
Asp	9·0 (10)	9·5 (10)	9·9 (9)	9	9–10	10	10
Thr	8·9 (10E)	10·2 (10E)	10·9 (9E)	9	10	11	10
Ser	13·7 (10E)	14·6 (10E)	16·0 (9E)	14	15	16	15
Glu	37·9 (10)	36·8 (10)	36·4 (9)	38	36–37	36–37	38–39
Pro	34·5 (10)	32·6 (9)	33·6 (9)	32–35§	32–33	33–34	34
Gly	5·0 (10)‖	5·0 (9)‖	5·0 (8)‖	5	5	5	5
Ala	5·2 (9)	5·8 (9)	6·6 (9)	5¶	6	7	7
Val	18·9 (7)**	17·2 (7)	17·5 (7)	19	17	17–18	19
Met	5·8 (10)	5·1 (10)	4·9 (9)	6	5	5	6
Ile	9·7 (7)**	9·5 (8)	9·8 (7)	10	10	10	11
Leu	21·6 (10)	20·0 (10)	20·1 (9)	21–22	20	20	21
Tyr	4·0 (10)	3·8 (10)	3·9 (2)††	4	4	4	5
Phe	8·8 (10)	8·2 (10)	8·2 (9)	9	8	8	9
Trp	1·0‡‡	1·0	1·0	1	1	1	1
P				5	5	3–4	4

* Derived from molar ratios and based on the presence of 5 glycine residues per molecule. The values listed are either averages or values extrapolated to zero times.

† The number in parentheses indicates the number of hydrolysates analyzed.

‡ The letter, E, means that progressive destruction of the amino acid occurred with increasing time of hydrolysis and that the value shown was obtained by linear extrapolation to zero time.

§ Our value of 34·5 residues of proline in β-casein B appears to be too high; analyses of another preparation have given 32–33 residues, in better agreement with values reported by Pion *et al.* (1965) and by Peterson *et al.* (1966).

‖ From which all other values were derived.

¶ Although the difference in alanine content between β-B and β-D is only 1·4 residues, we believe that our data strongly suggest the figures of 5, 6 and 7 listed.

** Results from 24-hr hydrolysates were not included in calculating results for valine and isoleucine.

†† The figure listed is the average of duplicate results of analyses of 24-hr hydrolysates; unaccountably high losses of tyrosine occurred on longer hydrolysis of β-casein D.

‡‡ Determined by Dr. J. R. Spies by Procedure U of the modified Spies method (Spies, 1967).

the others in that it contains one more each of aspartic acid (or asparagine), threonine, isoleucine and tyrosine, and *two* more alanine residues per molecule containing 5 glycine residues; this β-C is apparently a slightly larger molecule, of about 213 amino acid acid residues, than the other β-caseins thus far examined. As will be discussed later, our β-casein C contains only 4, rather than 5, atoms of phosphorus per molecule. It is interesting that this β-C and β-D are alike in having 7 alanine residues per molecule, a number unusually high for β-caseins. Multiple small differences in content of other amino acids are, however, manifest.

Peptide patterns

Figure 3 shows single-dimension peptide patterns of the tryptic digests of β-caseins, B, B_Z, C and D. β-caseins B and B_Z are very similar in their peptide patterns except for an additional acidic peptide in β-B. The second dimension (ascending chromatography, not illustrated), verifies the presence of this peptide. This observation supports the conclusion that the two proteins, while similar in composition, are not identical.

The patterns for β-caseins C (Western) and β-D (Zebu) also included in Fig. 3 indicate that (a) the peptide maps of all β-caseins shown are similar, and (b) β-C and D clearly differ in the position of their acidic peptides while D completely lacks one basic peptide.

DISCUSSION

The observation that β-caseins B, obtained from the milks of Jersey and Zebu cattle, differed in amino acid composition, while surprising, was not unexpected, in view of the suggestion of many that variants at the various casein loci would be discovered which differ in uncharged amino acids (Thompson *et al.*, 1965). In fact the criterion of electrophoretic mobility as proof of identity must be supplemented with compositional and high-voltage electrophoretic analysis of proteolytic digests as, for example, in this study. Nevertheless, two notable exceptions to the observation that variants differing in uncharged amino acids are detected with difficulty can be cited; those involved the α_{s1}-caseins, D, differing from B by a Pro/Ser substitution (deKoning & Van Rooijen, 1967) and α_{s1}-A, the mobility of which by gel-electrophoresis appears to result from the deletion of a segment of the molecule (Thompson *et al.*, 1969). However, the observation that variants exist which, while identical in electrophoretic mobility, differ in neutral amino acids, should not be a deterrent to continued studies on delineating the origin of cattle based in part on milk protein polymorphisms.

One would hardly expect (excluding possible differences in amide nitrogen) that the net negative charge on the protein deduced from amino acid composition (Table 1) would cause β-casein D to migrate slower than B (or B_Z) upon alkaline gel-electrophoresis. β-D migrates like β-B upon acid gel-electrophoresis. In fact, the most likely explanation for this mobility difference is the lower number (3–4) of phosphorus atoms/24,000 Daltons. β-Casein C is another interesting

example (Thompson & Pepper, 1964) of a variant which contains one less phosphorus atom/molecule. Several possible explanations for the lower phosphorus content of β-D and/or C can be postulated. Peterson *et al.* (1957) showed that the phospho-peptide (P-P), obtained by tryptic hydrolysis of β-casein (pooled milk), possessed both N- and C-terminal arginine. Undoubtedly the phosphorus-rich peptide is located in the N-terminal region of the intact β-casein molecule. The D and C variants each lack one residue of arginine. If we presume that this arginine residue is deleted at the C-terminal end of the P-P and substituted with serine, for example, then it is postulated that (1) either the substitution has altered the protein backbone sufficiently to sterically hinder phosphorylation or (2) that the substitution of a hydroxy-amino acid has resulted in competitive inhibition of the phosphorylase for an adjacent hydroxy-amino acid, presumably serine. An alternate, though an admittedly less plausible, hypothesis is that the phosphorylase may itself be genetically defective and hence unable to complete phosphorylation of the molecule. This later suggestion does not seem tenable, however, since β-B_Z isolated from a B_ZD heterozygote contains the normal amount of phosphorus i.e. 5 g atoms/24,000 Daltons.

Examination of the amino acid data indicates that, at this time, assignment of triplet codons to amino acid substitutions in the β-caseins studied is fruitless. While one may cite the purity of samples as the cause of lack of simple amino acid substitutions, one need only cite the studies on α_{s1}-caseins to discount purity as an explanation. The most plausible explanations are (1) that mutations have occurred in two or more steps or (2) that variants like B_Z exist as intermediate mutants which are not detected by current methods of phenotyping.

Many of the preceding remarks, concerning the occurrence of several forms of β-casein B, apply as well to β-casein C (*Bos taurus*) for it is clear that β-C itself is polymorphic.

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Key Word Index—Amino acid composition of β-casein; β-casein; *Bos indicus*; *Bos taurus*; casein variants (B_Z, β-D, β-C, D).